11-21-05

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09/444281

Docket No.: 20343/1202359-US1
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Letters Patent of:
Jan Burian et al.

Patent No.: 6,946,261

Issued: September 20, 2005

For: EFFICIENT METHODS FOR PRODUCING ANTI-MICROBIAL CATIONIC PEPTIDES IN HOST CELLS

REQUEST FOR CERTIFICATE OF CORRECTION
PURSUANT TO 37 CFR 1.322 AND 37 CFR 1.323

Attention: Certificate of Correction Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Upon reviewing the above-identified patent, Patentee noted several Patent Office errors which should be corrected.

The following errors were not in the application as filed by applicant:

In the Application:

First Page Col. 1 (Foreign Patent Documents), Line 1, Delete "6/1902" and insert

--6/2002 --.

First Page Col. 1 (Other Publications), Line 3, After "pp" insert --.--.

Page 2 Col. 2 (Other Publications), Line 11, Delete Catiionic" and insert -- Cationic --.

Column 1, Line 61, Delete "et al", and insert -- et al.,--.

Column 2, Line 13 (Approx.) Delete "et **at**", and insert -- et al.,--.

Column 2, Line 14 (Approx.) Delete "et al," and insert -- et al., --.

Column 2, Line 14 (Approx.) Delete "Acad" and insert -- Acad. --.

Column 4, Line 23 (Approx.) After "purified" delete "1".

Column 5, Line 35 (Approx.) Delete "1 m/min" and insert --1 m**I**/min --.

Column 8, Line 46 (Approx.) Delete "L" before "3.".

Column 10, Line 10 (Approx.) Delete "antifingal" and insert -- antifungal --.

Column 10 (Table 1), Line 20 Col. 3, After "AVATVGQAAAIARG" insert -- * --.

Column 10 (Table 1), Line 22 Col. 3, After "AVATVGQAAAIARG" insert -- * --.

Column 10 (Table 1), Line 29 Col. 3 Delete "GIGSAILSAGKSALKGIAKGLAE" and insert -- GIGSAILSAGKSALKG**L**AKGLAE --.

Column 13, Line 4 (Excluding Table 1) After "(1992);"delete "Bulet et al," and insert

-- Bulet et al., --.

Column 13, Line 4 (Excluding Table 1) After "JBC" insert --**268:** --.

Column 13, Line 13, After "Lambert" delete "e**r** al." and insert -- e**t** al., --.

Column 16, Line 46, After "Microbiol" insert --.--.

Column 18, Line 31, Delete "JM105" and insert -- JM101--.

Column 19, Line 13 (Approx.) Delete SF21(AQTCC CRL 1711)" and insert

--sf9 (ATCC CRL 1711) --.

Column 21, Line 5 (Approx.) "pET21CBD96x11B7" and insert

-- pET21CBD96-2x11B7 --.

Column 21, Line 50 (Approx.) "MBI-11 B7" and insert -- MBI-11B7 --.

Column 22, Line 19 (Approx.) Delete "65" and insert --6S--.

Column 26, Line 9, Delete pET21 CBD96" and insert -- pET21CBD96 --.

Column 26, Line 16, After "the" delete "+".

Column 26, Line 22, After Delete "(SEQ ID NO:11)" and insert --(SEQ ID NO:1)--.

Column 26, Line 49, After "Then" delete "is".

Column 26, Line 54, Delete "250° F." and insert - - 250 µF --.

Column 27, Line 35, Insert "µl" before "reactions".

Column 27, Line 42, Delete "2 Iµl" and insert - - 2µl - -.

Column 27, Line 52, Delete "100 Pg/ml" and insert - - 100 µg/ml - -.

Column 27, Line 62, Delete "30C" and insert --30°C - -.

Column 29, Line 41, Delete "pET21 CBD96-11" and insert -- pET21CBD96-11--.

Column 29, Line 42, Delete "pET21 CBD96-2x11" and insert -- pET21CBD96-2x11--.

Column 30, Line 5, Delete "pET21 CBD96-10x11" and insert -- pET21CBD96-10x11--.

Column 30, Line 14, Delete "GGCG" and insert --GCCG --.

Column 30, Line 54, Delete "11B 7" and insert 11B7 --.

Column 31, Line 7, Delete "Avionic" and insert --Anionic--.

Column 31, Line 44, Delete "11 B7S" and insert --11B7S --.

Column 32, Line 65-66 Delete "100 p g/ml" and insert - - 100 µg/ml - -.

Column 33, Line 11, Delete "soon" and insert --so on. --.

Column 33, Line 22, After "fifteen" delete ".".

Column 34, Line 8, Delete "2µL" and insert - - 2µl - -.

Column 34, Line 27, Delete "5S-x11B7" and insert - - 5S-5x11B7 --.

Column 34, Line 37, Delete "Mb126" and insert - - MBI26 - -.

Column 34, Line 48, Delete "mbi11 B7" and insert - - mbi11B7 - -.

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Column 35, Line 9, Delete "MBI126" and insert - - MBI26 - -.

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Column 37, Line 56, Delete "5 mL/min." and insert - - 5 ml/min. - -.

Column 38, Line 43 (Approx.) Delete "PEPTEDES" and insert -- PEPTIDES --.

Column 38 (TABLE 3), Line 47 (Approx. Heading) Delete "293B1" and insert

--203B1 --.

Column 38 (TABLE 3), Col. 5, Row 3, Delete "32" and insert --8 --.

Column 38 (TABLE 3), Col. 6, Row 2, Below ">64" insert - - 32 - -.

Enclosed please find copies of Sheet 1 and 2 of the List of References, Marked up copies of the specification, pages 2, 3, 6, 8, 13, 14, 15, 17, 18, 20, 23, 24, 27, 28, 29, 35, 36, 37, 38, 41, 42, 43, 44, 46, 47, 48, 49, 50, 54, 55 & 56.

The following error was found in the application as filed by applicant. The errors now sought to be corrected is an inadvertent typographical error, the correction of which does not involve new matter or require reexamination.

Column 26, Line 21, After "pmol each of " delete "each of".

Transmitted herewith is a proposed Certificate of Correction effecting such amendment. Patentee respectfully solicits the granting of the requested Certificate of Correction.

The Commissioner is authorized to charge any deficiency of up to $300.00 or credit any excess in this fee to Deposit Account No. 04-0100. Enclosed please find a check for $100.00.

Dated: November , 2005

Respectfully submitted,

By _____

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UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO.	:	6,946,261
APPLICATION NO.	:	09/444,281
ISSUE DATE	:	September 20, 2005
INVENTOR(S)	:	Jan Burian et al.

It is certified that an error appears or errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

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{S:\20343\1202359us1\00574915.DOC }MAILING ADDRESS OF SENDER
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membrane, causing a loss of potassium ions, membrane depolarization, and a decrease in cytoplasmic ATP.

Since their *de novo* synthesis or release from storage sites can be induced rapidly, cationic peptides are particularly important in the initial phases of resistance to microbial invasion. Cationic peptides are also effective when administered as therapeutic agents. In the treatment of topical infection, for example, an α-helical magainin variant peptide has been shown to be effective against polymicrobic foot ulcer infections in diabetics, and a protegrin-derived peptide was found useful for treatment of oral polymicrobic ulcers in cancer patients (Hancock and Lehrer, *TIBTECH 16*:82, 1998). Efficacy against systemic infection has been shown with an α-helical peptide used to treat *Pseudomonas aeruginosa* peritoneal infection, a β-sheet protegrin against methicillin-resistant *Staphylococcus aureus* and against vancomycin-resistant *Enterococcus faecalis*, and extended-helix indolicidin against *Aspergillus* fungal infections (Gough *et al.*, *Infect. Immun. 64*:4922, 1996; Steinberg *et al.*, *Antimicrob. Agents Chemother. 41*:1738, 1997; and Ahmad *et al.*, *Biochim. Biophys. Acta 1237*:109, 1995). Therefore, naturally-occurring cationic peptides, and their synthetic variants, are valuable antimicrobial therapeutics.

A practical drawback in cationic peptide therapy is the lack of a cost effective, mass-production method of the agents. Typically, the isolation of cationic peptides from natural sources is not cost-effective, and does not apply to the production of engineered cationic peptide variants which may have increased efficacy. While chemical peptide synthesis can be used to manufacture either natural or engineered cationic peptides, this approach is very costly.

Therefore, alternate, more economical and efficient methods of synthesis are needed, such as *in vivo* synthesis in host cells using recombinant DNA methods. Researchers have attempted various methods for recombinant production of cationic peptides. For example, cationic peptides have been produced in bacteria, such as *E. coli* or *Staphylococcus aureus*, yeast, insect cells, and transgenic mammals (Piers *et al.*, *Gene 134*:7, 1993, Reichhart *et al.*, *Invertebrate Reprod. Develop. 21*:15, 1992, Hellers

et al., *Eur. J. Biochem.* *199*:435, 1991, and Sharma *et al.*, *Proc. Nat'l Acad. Sci. USA* *91*:9337, 1994).

Much attention has focused on production in *E. coli*, since those versed in the art are familiar with the fact that high productivity can be obtained in *E. coli* using the recombinant DNA technology. However, for small peptides it is often necessary to produce them as part of a larger fusion protein. In this technique the gene for the peptide is joined to that of a larger carrier protein and the fusion expressed as a single larger protein. Following synthesis the peptide must be cleaved from the fusion partner. There is an extensive body of literature on protein fusion, especially in the gene expression host *E. coli*. For example, a number of recombinant proteins have been produced as fusion proteins in *E. coli*, such as, insulin A and B chain, calcitonin, Beta-globin, myoglobin, and a human growth hormone (Uhlen and Moks, "Gene Fusions for Purposes of Expression, An Introduction" in *Methods in Enzymology* *185*:129-143 Academic Press, Inc. 1990). Nevertheless, recombinant gene expression from a host cell presents a number of technical problems, particularly if it is desired to produce large quantities of a particular protein. For example, if the protein is a cationic peptide, such peptides are very susceptible to proteolytic degradation, possibly due to their small size or lack of highly ordered tertiary structure. One approach to solving this problem is to produce recombinant cationic proteins in protease-deficient *E. coli* host cell strains (see, for example, Williams *et al.*, U.S. Patent No. 5,589,364, and WO 96/04373). Yet there is no general way to predict which protease-deficient strains will be effective for a particular recombinant protein.

In principle the recombinant DNA technique is straight forward. However, any sequence that interferes with bacterial growth through replication or production of products toxic to the bacteria, such as lytic cationic peptides, are problematic for cloning. Foreign peptide gene products that are unstable or toxic, like cationic peptides, can also be stabilized by expressing the peptides as part of a fusion protein comprising a host cell protein. For example, Callaway *et al.et al.*, *Antimicrob. Agents Chemother.* *37*:1614, 1993, expressed cecropin A in *E. coli* as a fusion peptide with a truncated portion of the L-ribulokinase gene product, Piers *et al.et al.*, *Gene*

To the extent an anionic spacer is included, such a spacer may have, 0, 1, 2, or more cysteine residues. Within certain embodiments, there can be more, the same number, or fewer anionic spacers than cationic peptides in the fusion construct. Within certain embodiments, the anionic spacer is smaller in size than the cationic peptide.

A wide variety of cleavage sites can be utilized, including for example, a methionine residue. In addition, a wide variety of promoters can be utilized, including for example the *lacP* promoter, *tacP* promoter, *trcP* promoter, *srpP* promoter, SP6 promoter, T7 promoter, *araP* promoter, *trpP* promoter, and λ promoter.

The present invention also provides methods for producing fusion proteins utilizing the above-described expression cassettes. Within one embodiment, such methods generally comprise the step of culturing a recombinant host cell containing an expression cassette, under conditions and for a time sufficient to produce the fusion protein. Representative examples of suitable host cells include yeast, fungi, bacteria (*e.g.*, *E. coli*), insect, and plant cells.

Once the fusion protein has been produced, it may be further purified and isolated. Further, the fusion protein may be cleaved into its respective components (*e.g.*, utilizing low pH, or, a reagent such as cyanogen bromide, 2-(2-nitrophenylsulphenyl)-3-methyl-3'-bromoindolenine, hydroxylamine, *o*-iodosobenzoic acid, Factor Xa, thrombin, enterokinase, collagenase, *Staphylococcus aureus* V8 protease, endoproteinase Arg-C, or trypsin).

Further, the fusion protein or cleaved cationic peptide may be purified utilizing a chromatographic method (*e.g.*, an anion chromatography column or resin). Within certain embodiments, the column can be charged with a base, and washed with water prior to loading the column with said cationic peptide. Within various embodiments, the column can be equilibrated with water and up to about 8 M urea. Moreover, the cationic peptide is solubilized in a solution comprising up to about 8 M urea. Within further embodiments, the cationic peptide is solubilized in a solution comprising a mild organic solvent, such as, for example, acetonitrile, or, an alcohol such as methanol or ethanol.

represent the multidomain clones fused to CBD carrier. The lower panels show the multi-domain clones carrier-free. The left panels show the whole cell lysates, where the right side panels show the inclusion bodies partitioning step. The major band in each lane represents the relevant multidomain protein and the "x" numbers appearing at the bottom of each lane indicate the number of the MBI- peptide copies. Numbers appearing along the left edge of the gels represents molecular weight standards (kD).

Figure 8 shows maps of portions of plasmids pET21-3s-5x11B7 and pET21-5s-7x11B7.

Figure 9 is a chromatogram of the Q-Sepharose chromatography step for cationic peptide purification, which monitors UV absorbance at 280 nm and conductivity.

Figure 10 is a schematic drawing that illustrates the construction of plasmids pET21CBD-X and pET21CBD-B.

Figure 11 is a graph showing the results of reverse-phase analysis of the Q-Sepharose chromatography leading peak, representing pure cationic peptide. In this study, a C8-column (4.6 x 10, Nova-Pak, Waters) was equilibrated with 0.1%TFA in water at 1 ml/min flow rate. Then 50 µl of Q-Sepharose chromatography leading peak material, diluted with 50 µl equilibration solution, was loaded on the column. Elution was performed with a 0-45% gradient of solution B (0.1% TFA, 99.9% Acetonitrile) at 1% increase B per min, then step to 100% B.

DETAILED DESCRIPTION OF THE INVENTION

1. Overview

As discussed above, a successful approach to stabilizing foreign peptide gene products which are inherently unstable or toxic is to express them fused to a protein which displays stability in the relevant host cell. In the case of small cationic peptides, however, production of a fusion protein will lead to a small portion of the desired peptide and an apparent low yield. A major gain in productivity and therefore economics of the process can be made if the fraction of desired peptide in the fusion protein is substantially greater. A favored route for this concept concerns expression of a fusion protein containing multiple sequential copies (a concatamer

domain protein" comprises a combination of preferably more than one "cationic peptide domain," and an equal, smaller or higher number of "anionic spacer peptide domains" with suitable cleavage sites for separating cationic peptide from the rest of the multi-domain protein. The multi-domain protein can be fused to a carrier protein to achieve higher expression and/or stability. If stability and expression level of multi-domain protein are satisfactory, there is no need to use a carrier protein. An "anionic spacer peptide component" comprises at least one anionic spacer peptide with a cleavage site. The "cumulative charge" of a cationic peptide component refers to the total charge of all cationic peptides that comprise the cationic peptide component. Similarly, the "cumulative charge" of an anionic spacer peptide component refers to the total charge of all anionic spacer peptides that comprise the anionic spacer peptide component.

As used herein, "antimicrobial activity" refers to the ability to kill or to prevent the growth of a microbe, or to kill or to prevent the growth of microbe-infected cells. The term "microbe" includes bacteria, fungi, yeast, algae, protozoa, and viruses. This term includes but will not be limited to all these interpretive descriptions of the biological activity of the cationic peptide.

3. Construction and Expression of Vectors Comprising Cationic Peptide Genes

a. Cationic Peptide Expression Vectors

The present invention contemplates the production of "cationic peptide," as that term is defined above. For example, suitable cationic peptides include but are not limited to, naturally occurring cationic peptides and analogs thereof, cecropins, normally made by lepidoptera (Steiner *et al.*, *Nature 292*:246, 1981) and diptera (Merrifield *et al.*, *Ciba Found. Symp. 186*:5, 1994), by porcine intestine (Lee *et al.*, *Proc. Nat'l Acad. Sci. USA 86*:9159, 1989), by blood cells of a marine protochordate (Zhao *et al.*, *FEBS Lett. 412*:144, 1997), synthetic analogs of cecropin A, melittin, and cecropin-melittin chimeric peptides (Wade *et al.*, *Int. J. Pept. Protein Res. 40*:429, 1992), cecropin B analogs (Jaynes *et al.*, *Plant Sci. 89*:43, 1993), chimeric cecropin A/B hybrids (Düring, *Mol. Breed. 2*:297, 1996), magainins (Zasloff, *Proc. Nat'l Acad. Sci USA 84*:5449, 1987), cathelin-associated antimicrobial peptides from leukocytes of

humans, cattle, pigs, mice, rabbits, and sheep (Zanetti *et al.*, *FEBS Lett. 374:*1, 1995), vertebrate defensins, such as human neutrophil defensins [HNP 1-4], paneth cell defensins of mouse and human small intestine (Oulette and Selsted, *FASEB J. 10:*1280, 1996; Porter *et al.*, *Infect. Immun. 65*:2396, 1997), vertebrate β-defensins, such as HBD-1 of human epithelial cells (Zhao *et al.*, *FEBS Lett. 368*:331, 1995), HBD-2 of inflamed human skin (Harder *et al.*, *Nature 387*:861, 1997), bovine β-defensins (Russell *et al.*, *Infect. Immun. 64*:1565, 1996), plant defensins, such as Rs-AFP1 of radish seeds (Fehlbaum *et al.*, *J. Biol. Chem. 269:*33159, 1994), α- and β-thionins (Stuart *et al.*, *Cereal Chem. 19*:288, 1942; Bohlmann and Apel, *Annu. Rev. Physiol. Plant Mol. Biol. 42*:227, 1991), γ-thionins (Broekaert *et al.*, *Plant Physiol. 108*:1353, 1995), the anti-fungal drosomycin (Fehlbaum *et al.*, *J. Biol. Chem. 269*:33159, 1994), apidaecins, produced by honey bee, bumble bee, cicada killer, hornet, yellow jacket, and wasp (Casteels *et al.*, *J. Biol. Chem. 269*:26107, 1994; Levashina *et al.*, *Eur. J. Biochem. 233*:694, 1995), cathelicidins, such as indolicidin from bovine neutrophils (Falla *et al.*, *J. Biol. Chem. 277*:19298, 1996), bacteriocins, such as nisin (Delves-Broughton *et al.*, *Antonie van Leeuwenhoek J. Microbiol. 69*:193, 1996), and the protegrins and tachyplesins, which have antifungal, antibacterial and antiviral activities (Tamamura *et al.*, *Biochim. Biophys. Acta 1163*:209, 1993; Aumelas *et al.*, *Eur. J. Biochem. 237*:575, 1996; Iwanga *et al.*, *Ciba Found. Symp. 186*:160, 1994). Illustrative cationic peptides are listed in Table 1.

TABLE 1

ILLUSTRATIVE CATIONIC PEPTIDES**

Group Name	Peptide	Sequence	Reference*
Abaecins	Abaecin	YVPLPNVPQPGRRPFPTFPGQG PFNPKIKWPQGY	Casteels *et al.* (1990)
Andropins	Andropin	VFIDILDKVENAIHNAAQVGIG FAKPFEKLINPK	Samakovlis *et al.* (1991)
Apidaecins	Apidaecin IA	GNNRPVYIPQPRPPHPRI	Casteels *et al.* (1989)
	Apidaecin IB	GNNRPVYIPQPRPPHPRL	Casteels *et al.* (1989)
	Apidaecin II	GNNRPIYIPQPRPPHPRL	Casteels *et al.* (1989)
AS	AS-48	7.4 kDa	Galvez *et al.* (1989)
Bactenecins	Bactenecin	RLCRIVVIRVCR	Romeo *et al.* (1988)

Group Name	Peptide	Sequence	Reference*
Bac	Bac5	RFRPPIRRPPIRPPFYPPFRPPIRP PIFPPIRPPFRPPLRFP	Frank *et al.* (1990)
	Bac7	RRIRPRPPRLPRPRPRPLPFPRP GPRPIPRPLPFPRPGPRPIPRPLP FPRPGPRPIPRP	Frank *et al.* (1990)
Bactericidins	Bactericidin B2	WNPFKELERAGQRVRDAVISA APAVATVGQAAAIARG*	Dickinson *et al.* (1988)
	Bactericidin B-3	WNPFKELERAGQRVRDAIISA GPAVATVGQAAAIARG	Dickinson *et al.* (1988)
	Bactericidin B-4	WNPFKELERAGQRVRDAIISA APAVATVGQAAAIARG*	Dickinson *et al.* (1988)
	Bactericidin B-5P	WNPFKELERAGQRVRDAVISA AAVATVGQAAAIARGG*	Dickinson *et al.* (1988)
Bacteriocins	Bacteriocin C3603	4.8 kDa	Takada *et al.* (1984)
	Bacteriocin IY52	5 kDa	Nakamura *et al.* (1983)
Bombinins	Bombinin	GIGALSAKGALKGLAKGLAZH FAN*	Csordas and Michl (1970)
	BLP-1	GIGASILSAGKSALKGLAKGLA EHFAN*	Gibson *et al.* (1991)
	BLP-2	GIGSAILSAGKSALKGLAKGLA EHFAN*	Gibson *et al.* (1991)
Bombolitins	Bombolitin BI	IKITTMLAKLGKVLAHV*	Argiolas and Pisano (1985)
	Bombolitin BII	SKITDILAKLGKVLAHV*	Argiolas and Pisano (1985)
BPTI	Bovine Pancreatic Trypsin Inhibitor (BPTI)	RPDFCLEPPYTGPCKARIIRYFY NAKAGLCQTFVYGGCRAKRN NFKSAEDCMRTCGGA	Creighton and Charles (1987)
Brevinins	Brevinin-1E	FLPLLAGLAANFLPKIFCKITRK C	Simmaco *et al.* (1993)
	Brevinin-2E	GIMDTLKNLAKTAGKGALQSL LNKASCKLSGQC	Simmaco *et al.* (1993)
Cecropins	Cecropin A	KWKLFKKIEKVGQNIRDGIIKA GPAVAVVGQATQIAK*	Gudmundsson *et al.* (1991)
	Cecropin B	KWKVFKKIEKMGRNIRNGIVK AGPAIAVLGEAKAL*	Xanthopoulos *et al.* (1988)
	Cecropin C	GWLKKLGKRIERIGQHTRDATI QGLGIAQQAANVAATARG*	Tryselius *et al.* (1992)
	Cecropin D	WNPFKELEKVGQRVRDAVISA GPAVATVAQATALAK*	Hultmark *et al.* (1982)
	Cecropin P_1	SWLSKTAKKLENSAKKRISEGI AIAIQGGPR	Lee *et al.* (1989)
Charybdtoxins	Charybdtoxin	ZFTNVSCTTSKECWSVCQRLH NTSRGKCMNKKCRCYS	Schweitz *et al.* (1989)
Coleoptericins	Coleoptericin	8.1 kDa	Bulet *et al.* (1991)
Crabrolins	Crabrolin	FLPLILRKIVTAL*	Argiolas and Pisano (1984)
α-Defensins	Cryptdin 1	LRDLVCYCRSRGCKGRERMN GTCRKGHLLYTLCCR	Selsted *et al.* (1992)
	Cryptdin 2	LRDLVCYCRTRGCKRRERMN GTCRKGHLMYTLCCR	Selsted *et al.* (1992)
	MCP1	VVCACRRALCLPRERRAGFCRI RGRIHPLCCRR	Selsted *et al.* (1983)

Group Name	Peptide	Sequence	Reference*
	Magainin II	GIGKFLHSAKKFGKAFVGEIM NS*	Zasloff (1987)
	PGLa	GMASKAGAIAGKIAKVALKAL *	Kuchler *et al.* (1989)
	PGQ	GVLSNVIGYLKKLGTGALNAV LKQ	Moore *et al.* (1989)
	XPF	GWASKIGQTLGKIAKVGLKELI QPK	Sures and Crippa (1984)
Mastoparans	Mastoparan	INLKALAALAKKIL*	Bernheimer and Rudy (1986)
Melittins	Melittin	GIGAVLKVLTTGLPALISWIKR KRQQ	Tosteson and Tosteson (1984)
Phormicins	Phormicin A	ATCDLLSGTGINHSACAAHCL LRGNRGGYCNGKGVCVCRN	Lambert *et al.* (1989)
	Phormicin B	ATCDLLSGTGINHSACAAHCL LRGNRGGYCNRKGVCVRN	Lambert *et al.* (1989)
Polyphemusins	Polyphemusin I	RRWCFRVCYRGFCYRKCR*	Miyata *et al.* (1989)
	Polyphemusin II	RRWCFRVCYKGFCYRKCR*	Miyata *et al.* (1989)
Protegrins	Protegrin I	RGGRLCYCRRRFCVCVGR	Kokryakov *et al.* (1993)
	Protegrin II	RGGRLCYCRRRFCICV	Kokryakov *et al.* (1993)
	Protegrin III	RGGGLCYCRRRFCVCVGR	Kokryakov *et al.* (1993)
Royalisins	Royalisin	VTCDLLSFKGQVNDSACAANC LSLGKAGGHCEKGVCICRKTS FKDLWDKYF	Fujiwara *et al.* (1990)
Sarcotoxins	Sarcotoxin IA	GWLKKIGKKIERVGQHTRDAT IQGLGIAQQAANVAATAR*	Okada and Natori (1985b)
	Sarcotoxin IB	GWLKKIGKKIERVGQHTRDAT IQVIGVAQQAANVAATAR*	Okada and Natori (1985b)
Seminal plasmins	Seminalplasmin	SDEKASPDKHHRFSLSRYAKL ANRLANPKLLETFLSKWIGDR GNRSV	Reddy and Bhargava (1979)
Tachyplesins	Tachyplesin I	KWCFRVCYRGICYRRCR*	Nakamura *et al.* (1988)
	Tachyplesin II	RWCFRVCYRGICYRKCR*	Muta *et al.* (1990)
Thionins	Thionin BTH6	KSCCKDTLARNCYNTCRFAGG SRPVCAGACRCKIISGPKCPSD YPK	Bohlmann *et al.* (1988)
Toxins	Toxin 1	GGKPDLRPCIIPPCHYIPRPKPR	Schmidt *et al.* (1992)
	Toxin 2	VKDGYIVDDVNCTYFCGRNA YCNEECTKLKGESGYCQWASP YGNACYCKLPDHVRTKGPGR CH	Bontems *et al.* (1991)

*Argiolas and Pisano, *JBC* 259:10106 (1984); Argiolas and Pisano, *JBC* 260:1437 (1985); Banerjee and Hansen, *JBC* 263:9508 (1988); Bellamy *et al.*, *J. Appl. Bacter.* 73:472 (1992); Bernheimer and Rudy, *BBA* 864:123 (1986); Bohlmann *et al.*, *EMBO J.* 7:1559 (1988); Bontems *et al.*, *Science* 254:1521 (1991); Bulet *et al.*, *JBC* 266:24520 (1991); Bulet *et al.*, *Eur. J. Biochem.* 209:977 (1992); Bulet *et al.*, *JBC* 268:14893 (1993); Casteels *et al.*, *EMBO J.* 8:2387 (1989); Casteels *et al.*, *Eur. J. Biochem.* 187:381 (1990); Cociancich *et al.*, *BBRC* 194:17 (1993); Creighton and Charles, *J. Mol. Biol.* 194:11 (1987); Csordas and Michl, *Monatsh Chemistry* 101:82 (1970); Diamond

et al., *PNAS* 88:3952 (1991); Dickinson *et al.*, *JBC* 263:19424 (1988); Eisenhauer *et al.*, *Infect. and Imm.* 57:2021 (1989); Frank *et al.*, *JBC* 26518871 (1990); Fujiwara *et al.*, *JBC* 265:11333 (1990); Gálvez *et al.*, *Antimicrobial Agents and Chemotherapy* 33:437 (1989); Ganz *et al.*, *J. Immunol.* 143:1358 (1989); Gibson *et al.*, *JBC* 266:23103 (1991); Gudmundsson *et al.*, *JBC* 266:11510 (1991); Hanzawa *et al.*, *FEBS Letters* 269:413 (1990); Hastings *et al.*, *J. Bacteriology* 173:7491 (1991); Hultmark *et al.*, *Eur. J. Biochem.* 127:207 (1982); Hurst, *Adv. Appl. Micro.* 27:85 (1981); Kaletta *et al.*, *Archives of Microbiology* 152:16 (1989); Kokryakov *et al.*, *FEBS Letters* 327:231 (1993); Kuchler *et al.*, *Eur. J. Biochem.* 179:281 (1989); Lambert *et al.*, *PNAS* 86:262 (1989); Lee *et al.*, *PNAS* 86:9159 (1989); Lehrer *et al.*, *Cell* 64:229 (1991); Miyata *et al.*, *J. Biochem.* 106:663 (1989); Moore *et al.*, *JBC* 266:19851 (1991); Mor *et al.*, *Biochemistry* 30:8824 (1991); Muta *et al.*, *J. Biochem.* 108:261 (1990); Nakamura *et al.*, *JBC* 263:16709 (1988); Nakamura *et al.*, *Infection and Immunity* 39:609 (1983); Okada and Natori, *Biochem. J.* 229:453 (1985); Reddy and Bhargava, *Nature* 279:725 (1979); Reichhart *et al.*, *Eur. J. Biochem.* 182:423 (1989); Romeo *et al.*, *JBC* 263:9573 (1988); Samakovlis *et al.*, *EMBO J.* 10:163 (1991); Schmidt *et al.*, *Toxicon* 30:1027 (1992); Schweitz *et al.*, *Biochem.* 28:9708 (1989); Selsted *et al.*, *JBC* 258:14485 (1983); Selsted *et al.*, *JBC* 267:4292 (1992); Simmaco *et al.*, *FEBS Lett.* 324:159 (1993); Sures and Crippa, *PNAS* 81:380 (1984); Takada *et al.*, *Infect. and Imm.* 44:370 (1984); Tosteson and Tosteson, *Biophysical J.* 45:112 (1984); Tryselius *et al.*, *Eur. J. Biochem.* 204:395 (1992); Xanthopoulos *et al.*, *Eur. J. Biochem.* 172:371 (1988); Yamashita and Saito, *Infect. and Imm.* 57:2405 (1989); Zasloff, *PNAS* 84:5449 (1987).

** See also U.S Patent Nos. 4,822,608; 4,962,277; 4,980,163; 5,028,530; 5,096,886; 5,166,321; 5,179,078; 5,202,420; 5,212,073; 5,242,902; 5,254,537; 5,278,287; 5,300,629; 5,304,540; 5,324,716; 5,344,765; 5,422,424; 5,424,395; 5,446,127; 5,459,235; 5,464,823; 5,466,671; 5,512,269; 5,516,682; 5,519,115; 5,519,116; 5,547,939; 5,556,782; 5,610,139; 5,645,966; 5,567,681; 5,585,353; 5,589,568; 5,594,103, 5,610,139; 5,631,144; 5,635,479; 5,656,456; 5,707,855; 5,731,149; 5,714,467; 5,726,155; 5,747,449; 5,756,462; PCT Publication Nos. WO 89/00199; WO 90/11766; WO 90/11771; WO 91/00869; WO 91/12815; WO 91/17760; WO 94/05251; WO 94/05156; WO 94/07528; WO 95/21601; WO 97/00694; WO 97/11713; WO 97/18826; WO 97/02287; WO 98/03192; WO 98/07833; WO 98/07745; WO 98/06425 European Application Nos. EP 17785; 349451; 607080; 665239; and Japanese Patent/Patent Application Nos. 4341179; 435883; 7196408; 798381; and 8143596.

Nucleic acid molecules encoding cationic peptides can be isolated from natural sources or can also be obtained by automated synthesis of nucleic acid molecules or by using the polymerase chain reaction (PCR) with oligonucleotide primers having nucleotide sequences that are based upon known nucleotide sequences of cationic peptides. In the latter approach, a cationic peptide gene is synthesized using

Among the common amino acids, for example, a "conservative amino acid substitution" is illustrated by a substitution among amino acids within each of the following groups: (1) glycine, alanine, valine, leucine, and isoleucine, (2) phenylalanine, tyrosine, and tryptophan, (3) serine and threonine, (4) aspartate and glutamate, (5) glutamine and asparagine, and (6) lysine, arginine and histidine.

Nucleotide sequences encoding such "conservative amino acid" analogs can be obtained, for example, by oligonucleotide-directed mutagenesis, linker-scanning mutagenesis, mutagenesis using the polymerase chain reaction, and the like (see Ausubel (1995) at pages 8-10 to 8-22; and McPherson (ed.), *Directed Mutagenesis: A Practical Approach* (IRL Press 1991)). The antimicrobial activity of such analogs can be determined using a standard method, such as the assays described herein. Alternatively, a cationic peptide analog can be identified by the ability to specifically bind anti-cationic peptide antibodies. Typically, cationic peptide analogs should exhibit at least 50%, and preferably, greater than 70, 80 or 90%, of the activity of the corresponding naturally occurring cationic peptide.

Although one objective in constructing a cationic peptide variant may be to improve its activity, it may also be desirable to alter the amino acid sequence of a naturally occurring cationic peptide to enhance its production in a recombinant host cell. For example, a nucleotide sequence encoding a radish cationic peptide may include a codon that is commonly found in radish, but is rare for *E. coli*. The presence of a rare codon may have an adverse effect on protein levels when the radish cationic peptide is expressed in recombinant *E. coli*. Methods for altering nucleotide sequences to alleviate the codon usage problem are well known to those of skill in the art (see, for example, Kane, *Curr. Opin. Biotechnol. 6*:494, 1995, Makrides, *Microbiol. Rev. 60*:512, 1996, and Brown (Ed.), *Molecular Biology LabFax* (BIOS Scientific Publishers, Ltd. 1991), which provides a codon usage table on pages 245-253).

The present invention contemplates the use of "anionic spacer peptide" as that term is defined above. As described below, an illustrative anionic spacer peptide has the amino acid sequence of HEAEPEAEPIM where the methionine residue can be used as a cleavage site. Similar naturally occurring examples of anionic spacer peptides

protein domain, but include both anionic spacer peptide and cationic peptide components may include from five to more than 20 copies of a cationic peptide gene.

Preferably, cationic peptides are produced in prokaryotic host cells. Suitable promoters that can be used to express polypeptides in a prokaryotic host are well-known to those of skill in the art and for example include T4, T3, SP6 and T7 promoters recognized by specific phage RNA polymerases, the *int* , P_R and P_L promoters of bacteriophage lambda, the *trp*, *recA*, heat shock, *lacUV5*, *tac*, *lpp-lacSpr*, *phoA*, *lacP*, *tacP*, *trcP*, *srpP*, *araP*, and *lacZ* promoters of *E. coli*, promoters of *B. subtilis*, the promoters of the bacteriophages of *Bacillus*, *Streptomyces* promoters, the *bla* promoter of the *cat* promoter. Prokaryotic promoters have been reviewed by Glick, *J. Ind. Microbiol. 1*:277, 1987, Watson *et al.*, *Molecular Biology of the Gene*, *4th Ed.* (Benjamin Cummins 1987), and by Ausubel *et al.* (1995).

Preferred prokaryotic hosts include *E. coli*, *Bacillus subtilus*, and *Staphylococcus aureus*. Suitable strains of *E. coli* include BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pLysE, DH1, DH4I, DH5, DH5I, DH5IF', DH5IMCR, DH10B, DH10B/p3, DH11S, C600, HB101, JM101, JM105, JM109, JM110, K38, RR1, Y1088, Y1089, CSH18, ER1451, and ER1647 (see, for example, Brown (Ed.), *Molecular Biology Labfax* (Academic Press 1991)). Suitable strains of *Bacillus subtilus* include BR151, YB886, MI119, MI120, and B170 (see, for example, Hardy, "Bacillus Cloning Methods," in *DNA Cloning: A Practical Approach*, Glover (Ed.) (IRL Press 1985)). An illustrative strain of *Staphylococcus aureus* is RN4220 (Kreiswirth *et al.*, *Nature 305*:709, 1983). The present invention does not require the use of bacterial strains that are protease deficient.

An expression vector can be introduced into host cells using a variety of standard techniques including calcium phosphate transfection, microprojectile-mediated delivery, electroporation, and the like. Methods for introducing expression vectors into bacterial cells are provided by Ausubel (1995). Methods for expressing proteins in prokaryotic hosts are well-known to those of skill in the art (see, for example, Williams *et al.*, "Expression of foreign proteins in *E. coli* using plasmid vectors and purificatio

of specific polyclonal antibodies," in *DNA Cloning 2: Expression Systems*, *2nd Edition*,

Glover *et al.* (eds.), page 15 (Oxford University Press 1995); and Georgiou, "Expression of Proteins in Bacteria," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), page 101 (John Wiley & Sons, Inc. 1996)).

Cationic peptides can also be expressed in recombinant yeast cells. Promoters for expression in yeast include promoters from *GAL1* (galactose), *PGK* (phosphoglycerate kinase), *ADH* (alcohol dehydrogenase), *AOX1* (alcohol oxidase), *HIS4* (histidinol dehydrogenase), and the like. Many yeast cloning vectors have been designed and are readily available. These vectors include YIp-based vectors, such as YIp5, YRp vectors, such as YRp17, YEp vectors such as YEp13 and YCp vectors, such as YCp19. One skilled in the art will appreciate that there are a wide variety of suitable vectors for expression in yeast cells.

The baculovirus system provides an efficient means to express cationic peptide genes in insect cells. Suitable expression vectors are based upon the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), and contain well-known promoters such as *Drosophila* heat shock protein (hsp) 70 promoter, *Autographa californica* nuclear polyhedrosis virus immediate-early gene promoter (*ie-1*) and the delayed early *39K* promoter, baculovirus p10 promoter, and the *Drosophila* metallothionein promoter. Suitable insect host cells include cell lines derived from IPLB-*Sf*-21, a *Spodoptera frugiperda* pupal ovarian cell line, such as *Sf*9 (ATCC CRL 1711), *Sf*21AE, and *Sf*21 (Invitrogen Corporation; San Diego, CA), as well as *Drosophila* Schneider-2 cells. Established techniques for producing recombinant proteins in baculovirus systems are provided by Bailey *et al.*, "Manipulation of Baculovirus Vectors," in *Methods in Molecular Biology, Volume 7: Gene Transfer and Expression Protocols*, Murray (ed.), pages 147-168 (The Humana Press, Inc. 1991), by Patel *et al.*, "The baculovirus expression system," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), pages 205-244 (Oxford University Press 1995), by Ausubel (1995) at pages 16-37 to 16-57, by Richardson (ed.), *Baculovirus Expression Protocols* (The Humana Press, Inc. 1995), and by Lucknow, "Insect Cell Expression Technology," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 183-218 (John Wiley & Sons, Inc. 1996). Established methods for

domains. This method of construction of multi-domain genes allows the polymerization of any cationic peptide gene without changing its amino acid sequence. In initial studies, the MBI-11B7 cationic peptide was used.

Three distinct DNA cassettes specifying MBI-11B7 cationic peptide genes and a negatively charged peptide spacer were synthesized: 11B7-poly, anionic spacer, and 2x11B7-last (Figure 4), and cloned into appropriate plasmid vectors. Cassettes of 11B7-poly and spacer were linked together resulting in the 11B7poly-spacer cassette. The anionic spacer peptide and cationic peptide genes were separated by codons for Met to create sites for cleavage by cyanogen bromide (CNBr). Two codons, specifying Ala and a stop codon, were linked to the last 2x11B7 gene. The 2x11B7-last cassette was then cloned downstream of the gene encoding CBD96 in pET21CBD96 (Figure 5) resulting in plasmid pET21CBD96-2x11B7. This plasmid was later used in the construction of several fused multi-domain genes. The 11B7poly-spacer cassette was used in a serial cloning procedure which allowed polymerization of 11B7 genes into multi-domain fusion CBD96-spacer-poly11B7 proteins in the pET21CBD96 expression system (Figures 6). All multi-domain constructs containing *n* copies (where *n* = 3 to 30) of MBI-11B7 genes and (*n*-2) spacers were expressed at high levels. Examples of expression are shown in Figures 7. In order to accelerate the serial cloning procedure a polymerization cassette containing five 11B7 domains and five anionic spacer domains was prepared and used for construction of multidomain genes containing more than fifteen 11B7 domains (*i.e.*, 20 copies, 25, 30, etc.). This cassette has an anionic spacer domain at the end followed by a stop codon. Use of this cassette allowed construction of CBD96-based multi-domain systems containing equal numbers of 11B7 and spacer domains.

d. Illustrative Vectors Having a Nucleotide Sequence Encoding a Cationic Peptide With Anionic Spacers, But Lacking a Carrier Protein

One series of the multi-domain proteins comprises *n* times MBI-11B7 peptides and *n-2* anionic spacer peptides. When *n*=5, the molecular weight of the multi-domain protein equals 13.46 kDa, which should be sufficiently large for expression in *E. coli*. DNA fragments containing multi-domain genes of approximately this size were

excised from relevant plasmids using restriction endonucleases *Nde*I and *Hin*dIII and fused into plasmid containing specifically designed leader 11B7 domain. In *E. coli*, the first methionine in all proteins is translated as formyl-methionine which cannot be cleaved by CNBr. Accordingly, the carrier-free multi-domain proteins were modified in such a way that the first domain begins with M-T-M amino acids, allowing CNBr to cleave the first peptide at the second methionine and release authentic peptide. The relevant portions of plasmids pET21-3S-5x11B7 and pET21-5S-7x11B7 are shown in Figures 8. All of the carrier-free multi-domain constructs containing from 5 to 14 copies of MBI-11B7 genes were expressed at high levels as shown in Figure 7. In the same way, constructs were prepared containing an equal number of 11B7 and anionic spacer domains with a spacer sequence at the end. They were also expressed at high levels. The theoretical yield of the MBI-11B7 peptide, within experimentally obtained multi-domain proteins, can be seen in Table 2.

The invention also provides an additional example of another antimicrobial cationic peptide (MBI-26), twice the size of the peptide described above (MBI-11B7), consisting of 26 amino acids, where seven of them are basic amino acids. This peptide was artificially designed by a fusion between selected sequences of the natural antimicrobial cationic peptides cecropin and melittin. In the present invention, the last amino acid serine at the carboxy end was replaced with a methionine residue, which was used for release of the peptide from the multi-domain protein. The production of this peptide was obtained by recombinant synthesis in host cells, using the multi-domain protein method, as described above for MBI-11B7 peptide. Details are provided in Example 8.

TABLE 2

SUMMARY OF SUCCESSFULLY EXPRESSED CONSTRUCTS* AND THEIR THEORETICAL MBI-11B7CATIONIC PEPTIDE RATIO IN THE MULTI-DOMAIN PROTEINS, WITH AND WITHOUT CARRIER PROTEIN

Construct	Multi-domain Protein Mass (Da)	% Cationic Peptide per Multi-domain Protein (Da/Da)

Construct	Multi-domain Protein Mass (Da)	% Cationic Peptide per Multi-domain Protein (Da/Da)
With Carrier Protein	-	-
pET21CBD-11B7	21,249	8.9
pET21CBD-2x11B7	23,142	16.5
pET21CBD96-11B7	12,697	15.0
pET21CBD96-2x11B7	14,590	26.1
pET21CBD96-1S-3x11B7	17,718	32.3
pET21CBD96-2S-4x11B7	20,845	36.6
pET21CBD96-3S-5x11B7	23,973	39.8
pET21CBD96-4S-6x11B7	27,101	42.2
pET21CBD96-5S-7x11B7	30,228	44.2
pET21CBD96-6S-8x11B7	33,356	45.8
pET21CBD96-7S-9x11B7	36,484	47.1
pET21CBD96-8S-10x11B7	39,612	48.2
pET21CBD96-9S-11x11B7	42,739	49.1
pET21CBD96-10S-12x11B7	45,867	49.9
pET21CBD96-11S-13x11B7	48,995	50.6
pET21CBD96-12S-14x11B7	52,122	51.3
pET21CBD96-13S-15x11B7	55,250	51.8
pET21CBD96-18S-20x11B7	70,888	53.9
pET21CBD96-23S-25x11B7	86,527	55.1
pET21CBD96-28S-30x11B7	102,162	56.1
With equal spacers number	-	-
pET21CBD96-5S-5x11B7	26,282	36.3
pET21CBD96-10S-10x11B7	41,921	45.5
pET21CBD96-15S-15x11B7	57,559	48.9
Without Carrier Protein	-	-
pET21-3s-5x11B7-F	13,692	69.7
pET21-4s-6x11B7-F	16,820	68.1
pET21-5s-7x11B7-F	19,947	67.0
pET21-6s-8x11B7-F	23,075	66.2
pET21-7s-9x11B7-F	26,203	65.6
pET21-8s-10x11B7-F	29,330	65.1
pET21-9s-11x11B7-F	32,458	64.7
pET21-10s-12x11B7-F	35,586	64.4
pET21-11s-13x11B7-F	38,713	64.1
pET21-12s-14x11B7-F	41,841	63.9
pET21-19s-21x11B7-F	63,735	61.9
With equal spacers number	-	-
pET21-6s-6x11B7-F	19,129	59.9
pET21-11s-11x11B7-F	34,767	60.4
pET21-16s-16x11B7-F	50,405	60.6

* Examples of the expression can be seen in Figure 7.

Western blots, and other methods (see, generally, Harlow and Lane, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 1988)).

Expression vectors comprising the multi-domain fusion proteins described herein can be used to produce multi-domain fusion protein representing more than 25% of the total protein of a recombinant host cell. Since the multi-domain fusion proteins comprise multiple copies of a cationic peptide gene, the cationic peptide component of a fusion protein can be practically attained as more than 50% of the fusion protein.

The present invention, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

EXAMPLE 1

CONSTRUCTION OF PLASMIDS pET21CBD-X (B) AND pET21CBD96

Plasmid vector pET21a(+) (Novagen Corporation, USA), a T7 expression plasmid, was used as the core plasmid for all expression systems (Figures 1A and 10). The cellulose binding domain (CBD) from *Clostridium cellulovorans* was selected as a carrier protein for expression of antibacterial cationic peptides. Plasmid pET-CBD180 (Shpigel et al., *supra*) was used as the starting material (Figures 1B and 10). Restriction enzymes except *Vsp*I and *Nsi*I (Promega Corporation, USA), T4 DNA ligase and *Taq* polymerase were purchased from Pharmacia Biotech. The relevant part of CBD, including the T7 promoter of pET-CBD180, was amplified by PCR using 25 pmol each of each of the primers GCGT CCGG CGTA GAGG ATCG (SEQ ID NO:1) and CCGG GATC CAAT GTTG CAGA AGT AG (SEQ ID NO:2), 2 U of *Taq* DNA polymerase, corresponding reaction buffer (10x PCR reaction buffer: 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl, pH 9), 0.2 mM dNTPs (dATP, dGTP, dTTP and dCTP, Pharmacia Biotech) and 20 ng of heat-denatured pET-CBD180. PCR was

performed in MJ-Research PTC-100 Thermo-cycler in 50 µl reaction volume and 30 cycles of 94°C, 30 sec.; 55°C, 30 sec. and 72°C, 30 sec. A *Bam*HI restriction site (GGATCC) was incorporated at the 3'-end of the *cbd* gene to allow it to be cloned into pET21a(+). The *Bgl*II (AGATCT) or *Xba*I (TCTAGA) sites already present on pET-CBD180 were used to cut the 5'-end of the PCR fragment. One µg of PCR product was digested in a 100 µl reaction containing 1.5 x OPA (Pharmacia Biotech assay buffer One-Phor-All is supplied at 10x concentration: 100 mM Tris-acetate, pH 7.5; 100 mM magnesium acetate and 500 mM potassium acetate) and 10 U of *Bam*HI and 10 U of *Hind*III. Plasmid pET21a(+) was digested in the same way, in 2 x 50 µl reactions each containing 0.25 µg of plasmid DNA, 1.5x OPA and 2 U of *Bam*HI and *Hind*III each. Reactions were stopped by phenol/$CHCl_3$ extraction and ethanol precipitation. The resultant DNA pellets of digested pET21a(+) and relevant *cbd* and *cbd96* inserts were dissolved in 8 µl of water and mixed, then 2 µl of 10 mM ATP, 2 µl of 10x OPA and 2 U of T4 DNA ligase were added and reactions were incubated at 10°C for 1 hour. Then 2 µl of each ligation mixture were used to electroporate 40 µl of *E. coli* XL1 Blue (Promega Corporation) using a sterile Gene Pulser cuvette (0.2 cm electrode gap) and Gene Pulser electroporator apparatus (Bio-Rad Laboratories) set to 2.5 kV, 200 ohms and 250 µF. After an electroporation pulse, 1 ml of TB media (Maniatis, *et al.*, *Molecular Cloning: A Laboratory Manual, 2nd Ed.*, (Cold Spring Harbor Laboratory Press 1989) was added to the cell suspension and bacteria were incubated for 1 hour at 37°C with rigorous shaking. Then 10, 50 and 100 µl of cell suspension were plated on MacKonkey agar (BBL, Becton Dickinson and Company, USA) plates with 100 µg/ml of Ampicillin and incubated overnight at 37°C. The next day, several colonies were transferred to 2 ml of TB and cultivated at 37°C with vigorous shaking overnight. Then plasmid DNA was isolated and analyzed, including DNA sequencing by methods known to those skilled in the art. Positive clones contained plasmids pET21CBD-B or pET21CBD-X, respectively (Figure 10). Plasmid pET21CBD-X contains *lacO*, which improves the regulation of the T7 expression system. Both plasmids contain a stop codon downstream of *Bam*HI to allow expression of CBD180 protein. A T7 expression system was prepared in *E. coli* MC4100F (Strain MC4100F was prepared by mating

E. coli XL1Blue and *E. coli* MC4100; ATCC Number 35695) based on pET variants and pGP1-2, which carries the T7 RNA polymerase gene under a λR promoter controlled by cI857 thermo-sensitive repressor (Tabor and Richardson, *Biochemistry 82:*1074, 1985). CBD180 protein was expressed at high levels in both systems. Plasmid pET21CBD-X was used for subsequent work.

Plasmid pET21CBD96 (Figure 5) was prepared using the same PCR conditions and cloning procedures. In this experiment the carrier protein CBD180 was truncated to about 96 amino acids. Therefore a pair of PCR primers GCGT CCGG CGTA GAGG ATCG (SEQ ID NO:3) and ATAT GGAT CCAG ATAT GTAT CATA GGTT GATG TTGG GC (SEQ ID NO:4) was used to prepare the relevant DNA fragment encoding *cbd96* (Figure 5), which was then cloned into pET21a(+). Then again a T7 expression system was prepared in *E. coli* MC4100F based on plasmids pET21CBD96 and pGP1-2 and protein CBD96 was expressed at high levels. pET21CBD96 was used for most of the subsequent work.

EXAMPLE 2

CONSTRUCTION AND EXPRESSION OF CBD - MBI-11 FUSIONS

Sequences encoding all cationic peptides were designed from modified indolicidin, a natural anti-microbial peptide. Plasmids pET21CBD-X and pET21CBD96 (0.25 μg each) were digested with 2 U of *Bam*HI and 2 U of *Hind*III in 1.5x OPA in 50 μl reactions at 37°C for 1 hour. In the same way, a fragment encoding MBI-11 was digested (Example 4) using about 1 μg of DNA and 25U of *Bam*HI and *Hind*III each in a 100 μl reaction. Both reactions were stopped by phenol/$CHCl_3$ (Sigma-Aldrich Canada Ltd.) extraction and ethanol precipitation. The resultant DNAs of each vector and MBI-11 insert were dissolved in 8 μl of water and mixed, then 2 μl of 10 mM ATP, 2 μl of 10x OPA and 2 U of T4 DNA ligase were added and ligation reactions were incubated at 10°C for 1 hour. Then 2 μl of each ligation mixture was used to electroporate 40 μl of *E. coli* XL1 Blue using sterile Gene Pulser cuvettes (0.2 cm

electrode gap) and Gene Pulser electroporator apparatus set to 2.5 kV, 200 ohms and 250 μF. After an electroporation pulse, 1 ml of TB media was added to the cell suspension and bacteria were incubated for 1 hour at 37°C with rigorous shaking. Then 10, 50 and 100 μl of cell suspension were plated on MacKonkey agar plates with 100 μg/ml of Ampicillin (Sigma-Aldrich Canada Ltd.) and incubated overnight at 37°C. The next day, several colonies were transferred to 2 ml of TB and cultivated at 37°C with vigorous shaking overnight. Then plasmid DNA was isolated and analyzed, including DNA sequencing by methods known to those skilled in the art. Positive clones contained MBI-11 fused to CBD180 or CBD96. Expression strains of *E. coli* MC4100F harboring plasmids pGP1-2 and pET21CBD-11 or pET21CBD96-11 respectively were prepared by electroporation. Final strains were incubated overnight in 2 ml TB at 30°C with rigorous shaking and the next day 1 ml of cell suspension was diluted with the equal volume of fresh TB and cultivation temperature was increased to 42°C for a minimum of 2 hours. Samples of preinduced and induced cells were analyzed by SDS-PAGE. The level of expression of the fusion protein caring MBI-11or 2x MBI-11 gene was high and equal to expression of CBD180 or CBD96 alone.

EXAMPLE 3

EXPRESSION OF CBD FUSED POLYCATIONIC PEPTIDE TANDEM DOMAINS

This experiment was designed to test how many peptide genes in tandem can be fused to a carrier protein and expressed. It was necessary to create two DNA fragments encoding MBI-11, one for polymerization by DNA cloning and another one as the last gene in the tandem. Therefore, the original DNA fragment encoding MBI-11 peptide with COOH end was modified in order to create the last gene in tandem (Example 4) and a new gene was designed for a specific cloning procedure, which allowed construction of multiple tandem peptide genes fused to CBD180 or CBD96 carrier proteins genes (Example 4). The cloning procedure resulted in addition of an extra isoleucine to the MBI-11 tandem sequences. Therefore in order to produce

reaction buffer: 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl pH 9), 0.2 mM dNTPs (dATP, dGTP, dTTP and dCTP), 25 pmol of each primer and 50 pmol of template oligonucleotide resulting in 114 bp dsDNA MBI-11-BE fragment. This fragment was cloned as a *Bam*HI - *Eco*RI insert into vector pBCKS(+) resulting in pBCKS-11BE.

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D. POLYMERIZATION CLONING PROCEDURE

The copy of MBI-11 designed for the polymerization cloning procedure was cloned into pET21CBD96-11 resulting in pET21CBD96-2x11. pBCKS-11BE was digested with 2 U of *Bam*HI and *Vsp*I in 2x OPA in 50 µl reactions at 37°C for 1 hour and pET21CBD96-11 was digested with 2 U of *Bam*HI and *Nde*I in 2x OPA in a 50 µl reaction at 37°C for 1 hour. Reactions were stopped by phenol/$CHCl_3$ extraction and ethanol precipitation. The resulting DNA pellets were dissolved in 8 µl of water each and mixed, then 2 µl of 10 mM ATP, 2 µl of 10x OPA and 2 U of T4 DNA ligase were added and reactions were incubated at 10°C for 1 hour. Then 2 µl of the ligation mixture was used to electroporate 40 µl of *E. coli* XL1 Blue using Gene Pulser cuvettes (0.2 cm electrode gap) and Gene Pulser (Bio-Rad Laboratories) set to 2.5 kV, 200 ohms and 250 µF. After an electroporation pulse, 1 ml of TB media was added to the cell suspension and bacteria were incubated 1 hour at 37°C with rigorous shaking. Then 10, 50 and 100 µl of cell suspension were plated on MacKonkey agar plates with 100 µg/ml of Ampicillin and incubated overnight at 37°C. The next day, several colonies were transferred to 2 ml of TB and cultivated at 37°C with vigorous shaking overnight. Then plasmid DNA was isolated and analyzed, including DNA sequencing by methods known to those skilled in the art. Positive clones contained pET21CBD96-2x11. The ligation of compatible *Vsp*I and *Nde*I cohesive ends resulted to elimination of both restriction sites. At the same time, the insertion of the mbi-11be cassette introduced a new *Nde*I site, which allowed repetition of the cloning procedure and insertion of another mbi-11be. This procedure could be repeated theoretically without limitation. In this particular case the serial cloning was repeated nine times and constructs up to pET21CBD96-10x11 were prepared.

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EXAMPLE 5

SYNTHESIS OF DNA CASSETTES FOR CONSTRUCTION OF FUSED AND UNFUSED MULTI-DOMAIN EXPRESSION SYSTEMS

A. ***SYNTHESIS OF MBI 2x11B7-LAST CASSETTE***

An oligonucleotide CGCC AGGG TTTT CCCA GTCA CGAC GGAT CCGT CTCA TATG ATTC TGCG TTGG CCGT GGTG GCCG TGGC GTCG CAAA ATGA TTCT GCGT TGGC CGTG GTGG CCGT GGCG TCGC AAAA TGGC GGCC TAAG CTTC GATC CTCT ACGC CGGA CGC (SEQ ID NO:14) was synthesized and used as a template for PCR using primers CGCC AGGG TTTT CCCA GTCA CGAC (SEQ ID NO:15) and GCGT CCGG CGTA GAGG ATCG (SEQ ID NO:16). The PCR was performed in MJ-Research PTC-100 Thermo-cycler in a 50 µl reaction volume with 30 cycles of 94°C, 30 sec.; 55°C, 30 sec. and 72°C, 30 sec. 2 U of *Taq* DNA polymerase, corresponding reaction buffer (10x PCR reaction buffer: 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl pH 9), 0.2 mM dNTPs (dATP, dGTP, dTTP and dCTP), 25 pmol of each primer and 50 pmol of template oligonucleotide resulting in 151 bp dsDNA MBI-11 fragment. The PCR product was purified by phenol/$CHCl_3$ extraction and ethanol precipitation. The resulting DNA was dissolved in 100 µl 1x OPA , 20U of *Bam*HI and 20U of *Hind*III and the reaction was incubated at 37°C for 2 hours. The vector pBCKS(+) (0.25 µg) was digested in the same way. Both reactions were stopped by phenol/$CHCl_3$ extraction and ethanol precipitation. The resultant DNAs of each vector and MBI-11 insert were dissolved in 8 µl of water and mixed, then 2 µl of 10 mM ATP, 2 µl of 10x OPA and 2 U of T4 DNA ligase were added and ligation reactions were incubated at 10°C for 1 hour. Then 2 µl of each ligation mixture was used to electroporate 40 µl of *E. coli* XL1 Blue using a sterile Gene Pulser cuvette (0.2 cm electrode gap) and Gene Pulser electroporator apparatus set to 2.5 kV, 200 ohms and 250 µF. After an electroporation pulse, 1 ml of TB media was added to the cell suspension and bacteria were incubated 1 hour at 37°C with rigorous shaking. Then 10, 50 and 100 µl of cell suspension were plated on MacKonkey agar plates with 100 µg/ml of Ampicillin and incubated overnight at 37°C. The next day,

several colonies were transferred to 2 ml of TB and cultivated at 37°C with vigorous shaking overnight. Then plasmid DNA was isolated and analyzed, including DNA sequencing by methods known to those skilled in the art. The resulting plasmid was pBCKS-2x11B7. The insert was later recloned into pBCKS-V resulting in pBCKS-V-2x11B7.

B. SYNTHESIS OF MBI-11B7-POLY CASSETTE

An oligonucleotide CGCC AGGG TTTT CCCA GTCA CGAC GGAT CCGT CTCA TATG ATTC TGCG TTGG CCGT GGTG GCCG TGGC GTCG CAAA ATGC ATAA GCTT CGAT CCTC TACG CCGG ACGC (SEQ ID NO:17) was synthesized and used as a template for PCR using primers CGCC AGGG TTTT CCCA GTCA CGAC (SEQ ID NO:18) and GCGT CCGG CGTA GAGG ATCG (SEQ ID NO:19). The PCR was performed in MJ-Research PTC-100 Thermo-cycler in a 50 µl reaction volume with 30 cycles of 94°C, 30 sec.; 55°C, 30 sec. and 72°C, 30 sec., 2 U of *Taq* DNA polymerase, corresponding reaction buffer (10x PCR reaction buffer: 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl pH 9), 0.2 mM dNTPs (dATP, dGTP, dTTP and dCTP), 25 pmol of each primer and 50 pmol of template oligonucleotide resulting in a 112 bp dsDNA MBI-11 fragment. The resulting DNA fragment was cloned into pTZ18R (Pharmacia Biotech) as a *Bam*HI-*Hind*III fragment as described in paragraph (A) resulting in plasmid pTZ18R-11B7poly.

C. SYNTHESIS OF ANIONIC SPACER CASSETTE

An oligonucleotide CGCC AGGG TTTT CCCA GTCA CGAC GGAT CCGT CTAT GCAT GAAG CGGA ACCG GAAG CGGA ACCG ATTA ATTA AGCT TCGA TCCT CTAC GCCG GACG C (SEQ ID NO:20) was synthesized and used as a template for PCR using primers CGCC AGGG TTTT CCCA GTCA CGAC (SEQ ID NO:21) and GCGT CCGG CGTA GAGG ATCG (SEQ ID NO:22). The PCR was performed in MJ-Research PTC-100 Thermo-cycler in a 50 µl reaction volume with 30 cycles of 94°C, 30 sec.; 55°C, 30 sec. and 72°C, 30 sec., 2 U of *Taq* DNA polymerase, corresponding reaction buffer (10x PCR reaction buffer: 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl pH 9), 0.2 mM dNTPs (dATP, dGTP, dTTP and

dCTP), 25 pmol of each primer and 50 pmol of template oligonucleotide resulting in a 97 bp dsDNA MBI-11 fragment. The resulting DNA fragment was cloned into pBCKS-V as a *Bam*HI-*Hind*III fragment as described in paragraph (A), resulting in plasmid pBCKS-V-S.

D. ***SYNTHESIS OF MBI-11B7-FIRST CASSETTE***

An oligonucleotide CGCC AGGG TTTT CCCA GTCA CGAC GGAT CCGT CTCA TATG ACTA TGAT TCTG CGTT GGCC GTGG TGGC CGTG GCGT CGCA AAAT GCAT AAGC TTCG ATCC TCTA CGCC GGAC GC (SEQ ID NO:23) was synthesized and used as a template for PCR using primers CGCC AGGG TT TT CCCA GTCA CGAC (SEQ ID NO:24) and GCGT CCGG CGTA GAGG ATCG (SEQ ID NO:25). The PCR was performed in MJ-Research PTC-100 Thermo-cycler in a 50 µl reaction volume with 30 cycles of 94°C, 30 sec.; 55°C, 30 sec. and 72°C, 30 sec, 2 U of *Taq* DNA polymerase, corresponding reaction buffer (10x PCR reaction buffer: 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl, pH 9), 0.2 mM dNTPs (dATP, dGTP, dTTP and dCTP), 25 pmol of each primer and 50 pmol of template oligonucleotide resulting in a 114 bp dsDNA MBI-11 fragment. The resulting DNA fragment was cloned into pBCKS-V-S as a *Bam*HI- *Nsi*I fragment basically as described in paragraph (A), resulting in plasmid pBCKS-V-11B7S-F. The only exception was that 2x OPA was used in the restriction enzyme digest reaction.

E. ***CONSTRUCTION OF PLASMID PBCKS-V***

Plasmid pBCKS-V was prepared from pBCKS(+). The goal was to eliminate all *Vsp*I restriction sites from the original plasmid and use the resulting plasmid for cloning of some of DNA cassettes.

About 1 µg of pBCKS(+) was digested with *Vsp*I (Promega) in 50 µl reaction using 1x OPA. The reaction was stopped by phenol/$CHCl_3$ extraction and ethanol precipitation. The resulting DNA was dissolved in 50 µl of 1x OPA, 0.2 mM dNTPs and 1 U of Klenow polymerase. The reaction was incubated at 30°C, for 30 min. and then stopped by phenol/$CHCl_3$ extraction and ethanol precipitation. DNA was then dissolved in 50 µl of 1x OPA, 0.5 mM ATP and 15U of T4 DNA ligase and the reaction

of Ampicillin and incubated overnight at 37°C. The next day several colonies were transferred to 2 ml of TB and cultivated at 37°C with vigorous shaking overnight. Then plasmid DNA was isolated and analyzed, including DNA sequencing by methods known to those skilled in the art. Positive clones pET21CBD96-2x11B7 contained tandem MBI-11 genes fused to *cbd96*.

B. THE USE OF SERIAL CLONING PROCEDURE FOR CONSTRUCTION OF FUSED MULTI-DOMAIN PLASMIDS

The idea of the serial cloning procedure is that the insertion of the *Bam*HI- MBI-11B7-P-*Vsp*I cassette into the *Bam*HI - *Nde*I sites of pET21CBD96-2x11B7 and subsequent multi-domain clones always eliminates the original *Nde*I site by *Nde*I/*Vsp*I ligation and a new *Nde*I site is introduced with each insertion, which together with *Bam*HI is used for the next cycle of cloning.

Plasmid pET21CBD96-2x11B7 (0.25 μg) was digested with 2 U of *Bam*HI and *Nde*I in 2x OPA in 50 μl reaction at 37°C for 1 hour. Plasmid pBCKS-V-11B7S (2.5 μg) was digested in a 100 μl reaction with 20 U of *Bam*HI and *Vsp*I in 2x OPA at 37°C for 1 hour. Both reactions were stopped by phenol/$CHCl_3$ extraction and ethanol precipitation. The resulting DNAs were dissolved in 8 μl of water and mixed, then 2 μl of 10 mM ATP, 2 μl of 10x OPA and 2 U of T4 DNA ligase were added and the ligation reaction was incubated at 10°C for 1 hour. Then 2 μl of the ligation mixture were used to electroporate 40 μl of *E. coli* XL1 Blue using a sterile Gene Pulser cuvette (0.2 cm electrode gap) and Gene Pulser electroporator apparatus set to 2.5 kV, 200 ohms and 250 μF. After an electroporation pulse, 1 ml of TB media was added to the cell suspension and bacteria were incubated 1 hour at 37°C with rigorous shaking. Then 10, 50 and 100 μl of cell suspension were plated on MacKonkey agar plates with 100 μg/ml of Ampicillin and incubated overnight at 37°C. The next day, several colonies were transferred to 2 ml of TB and cultivated at 37°C with vigorous shaking overnight. Then plasmid DNA was isolated and analyzed, including DNA sequencing by methods known to those skilled in the art. Positive clones pET21CBD96-1s-3x11B7 contained three MBI-11 units with one spacer fused to *cbd96*. This was the first cycle of the serial cloning. In the next cycle pET21CBD96-1s-3x11B7 and pBCKS-V-11B7S were used

and cloning was repeated resulting in pET21CBD96-2s-4x11B7. Then pET21CBD96-2s-4x11B7 and pBCKS-V-11B7S were used for the next cloning resulting in pET21CBD96-3s-5x11B7 and so on.

In order to accelerate the serial cloning procedure plasmid pBCKS-V-5x11B7S was prepared and each cloning cycle would add five 11B7S domains. First the 11B7S insert of pBCKS-V-11B7S was recloned into pTZ18R, resulting in pTZ18R-11B7S. Then this plasmid was used as the donor of the 11B7S domain for the serial cloning into pBCKS-V-11B7S using the *Bam*HI-*NdeI*/*Vsp*I strategy. The serial cloning procedure was repeated four times resulting in pBCKS-V-5S-5x11B7S. The 5S-5x11B7 cassette was then used for construction of CBD96-fused systems containing more than fifteen 11B7 domains and also CBD96-fused multidomain systems with equal numbers of 11B7 and anionic spacer domains (Table 2).

The cassette 5S-5x11B7 of pBCKS-V-5S-5x11B7 with anionic spacer domain at the end was cloned into pET21CBD96 using *Bam*HI and *Kpn*I restriction enzymes resulting in pET21CBD96-5S-5x11B7. In the second cloning cycle the same cassette was ligated as *Bam*HI-*Vsp*I fragment of pBCKS-V-5x11B7S into *Bam*HI-*Nde*I sites of pET21CBD96-5S-5x11B7 resulting in pET21CBD96-10S-10x11B7. This can be repeated several times to receive constructs with 15, 20, 25 etc. 11B7 domains and equal numbers of anionic spacer domains. Conditions for restriction enzymes, ligation, electroporation and analysis of recombinant plasmids are described above.

EXAMPLE 7

CONSTRUCTION OF UNFUSED MULTI-DOMAIN EXPRESSION SYSTEMS

In *E. coli,* the first amino acid in all proteins is f-methionine. However, this amino acid is not cleaved by CNBr, which means that one peptide domain released from a multi-domain protein would start with f-methionine. The solution was to create a modified MBI-11 cassette encoding f-methionine and methionine in tandem at the beginning of the peptide, so the second one would be cleaved by CNBr. The result was

the synthesis of the special first domain in multi-domain genes, cassette MBI-11B7F, encoding MTM amino acids at the beginning. This domain was fused to the spacer domain in pBCKS-V-S resulting in plasmid pBCKS-V-11B7S-F.

Plasmid pBCKS-V-11B7S-F and the relevant pET21CBD96-multi-domain-11B7 plasmids were used for construction of unfused multi-domain MBI-11B7 genes. Multi-domain genes were liberated from *cbd96* by *Nde*I - *Xho*I digestion and cloned into the *Vsp*I - *Xho*I sites of pBCKS-V-11B7S-F downstream of the 11B7S insert. This created a line of unfused multi-domain 11B7 genes in plasmid pBCKS-V. These genes were then recloned as *Nde*I - *Xho*I fragments into pET21a(+) resulting in a series of pET plasmids capable of expression of multi-domain proteins using the T7 promoter system.

Plasmid pBCKS-V-11B7S-F (0.25 μg) was digested with 2 U of *Nde*I and *Xho*I in 2x OPA in several 50 μl reactions at 37°C for 1 hour. Relevant plasmids pET21CBD96-multidomain-11B7 (2.5 μg) were digested in 100 μl reactions with 20 U of *Nde*I and *Xho*I in 2x OPA at 37°C for 1 hour. All reactions were stopped by phenol/$CHCl_3$ extraction and ethanol precipitation. The resultant vector and insert DNAs were dissolved in 8 μl of water and mixed, then 2 μl of 10 mM ATP, 2 μl of 10x OPA and 2 U of T4 DNA ligase were added and ligation reactions were incubated at 10°C for 1 hour. Then 2 μl of each ligation mixture was used to electroporate 40 μl of *E. coli* XL1 Blue using a sterile Gene Pulser cuvette (0.2 cm electrode gap) and Gene Pulser electroporator apparatus set to 2.5 kV, 200 ohms and 250 μF. After an electroporation pulse, 1 ml of TB media was added to the cell suspension and bacteria were incubated 1 hour at 37°C with rigorous shaking. Then 10, 50 and 100 μl of cell suspension were plated on MacKonkey agar plates with 100 μg/ml of Ampicillin and incubated overnight at 37°C. The next day several colonies were transferred to 2 ml of TB and cultivated at 37°C with rigorous shaking overnight. Then plasmid DNA was isolated and analyzed, including DNA sequencing by methods known to those skilled in the art. Positive clones contained pET21-multidomain-11B7 plasmids containing 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 21 MBI-11B7 domains.

In the same way, constructs were prepared containing equal numbers of 11B7 and anionic spacer domains. By way of illustration: pET21CBD96-5S-5x11B7 was digested with *Bam*HI and *Xho*I (or *Hin*dIII) and fragment 5S-5x11B7 was ligated into *BamHI-XhoI* (or *Hin*dIII) of pBCKS-V-11B7S-F resulting in pBCKS-V-6S-6x11B7. The *Bam*HI-6S-6x11B7-*Xho*I cassette of pBCKS-V-6S-6x11B7 was then recloned into *Bam*HI-*Xho*I of pET21a(+) resulting in pET21-6S-6x11B7. All cloning procedures and clone analysis are described above.

EXAMPLE 8

CONSTRUCTION OF FUSED MULTIDOMAIN MBI26 EXPRESSION SYSTEMS

In our previous work we solved all major problems connected to the construction of multidomain cationic peptide expression systems. This example demonstrates we were able to simplify the process, especially the need for synthesis of multiple specific DNA cassettes; only one mbi26 cassette was prepared and used at the first and last position as well as for the serial cloning procedure. Plasmids pET21CBD96-1s-26 and pET21CBD96-2s-2x26 were prepared. We tested expression of a combination of mbi26 and mbi11B7 domains. We performed two cloning cycles, inserting mbi26S cassettes into pET21CBD96-1S-3x11B7, resulting in pET21CBD96-26S-3x11B7 and pET21CBD96-2x26S-3x11B7. Both constructs expressed the combined mbi26-11B7 multidomain proteins at good levels.

A. SYNTHESIS OF UNIVERSAL MBI26 DOMAIN

An oligonucleotide CGCC AGGG TTTT CCCA GTCA CGAC GGAT CCGT CTCA TATG ACCA TGAA ATGG AAAT CTTT CATC AAAA AACT GACC TCTG CTGC TAAA AAAG TTGT TACC ACCG CTAA ACCG CTGA TCTC TATG CATG CTTA AGCT TCGA TCCT CTAC GCCG GACG C (SEQ ID NO: 26) was synthesized and used as a template for PCR using primers CGCC AGGG TTTT CCCA GTCA CGAC (SEQ ID NO:18) and GCGT CCGG CGTA GAGG ATCG (SEQ

ID NO:19). PCR was performed in an MJ-Research PTC-100 Thermo-cycler in a 50 µl reaction volume with 30 cycles of 94°C, 30 sec.; 55°C, 30 sec. and 72°C, 30 sec., 2 U of *Taq* DNA polymerase, corresponding reaction buffer (10x PCR reaction buffer: 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl pH 9), 0.2 mM dNTPs (dATP, dGTP, dTTP and dCTP), 25 pmol of each primer and 50 pmol of template oligonucleotide resulting in a 112 bp dsDNA MBI26 fragment. The resulting DNA fragment was cloned into pTZ18R as a *Bam*HI-*Hind*III fragment as described in Example 2, paragraph (A) resulting in plasmid pTZ18R-26GT. After verification of DNA sequence, the *Bam*HI-*Hind*III mbi26 fragment was recloned into pBCKS(+) resulting in pBCKS-26GT.

B. ***CONSTRUCTION OF MBI26 FUSED MULTIDOMAIN SYSTEM***

The first step in construction was a direct fusion of the mbi26 cassette to cbd96 in pET21CBD96. Plasmids pET21CBD96 (0.25 µg) and pBCKS-26GT (2.5 µg) were digested with *Bam*HI and *Hind*III in 1.5x OPA (50 µl reaction volume) at 37°C for 1 hour using 2 U of each restriction enzyme and 20U of each enzyme respectively. Both reactions were stopped by phenol/$CHCl_3$ extraction and ethanol precipitation. Each resulting DNA was dissolved in 8 µl of water; the two were mixed together with 2 µl of 10 mM ATP, 2 µl of 10x OPA and 2 U of T4 DNA ligase and the ligation reaction was incubated at 10°C for 1 hour. 2 µl of the ligation mixture was used to electroporate 40 µl of *E. coli* XL1 Blue using a sterile Gene Pulser cuvette (0.2 cm electrode gap) and Gene Pulser electroporator apparatus set to 2.5 kV, 200 ohms and 250 µF. After an electroporation pulse, 1 ml of TB media was added to the cell suspension and bacteria were incubated 1 hour at 37°C with rigorous shaking. Then 10, 50 and 100 µl of cell suspension were plated on MacKonkey agar plates with 100 µg/ml of Ampicillin and incubated overnight at 37°C. The next day several colonies were transferred to 2 ml of TB and cultivated at 37°C with rigorous shaking overnight. Then plasmid DNA was isolated and analyzed, including DNA sequencing by methods known to those skilled in the art. Positive clones pET21CBD96-26 contained the MBI-26 gene fused to *cbd96*.

The second step was preparation of a cassette for the serial cloning procedure. The mbi26 fragment of pTZ18R-26GT was cloned into pBCKS-V-S as a

EXAMPLE 12

FREE CATIONIC PEPTIDE PURIFICATION

The purification of the homoserine lactone form of MBI-11B7 peptide was performed on a BioSys™ 2000 chromatography work station (Beckman Instruments, Inc.), using Fast Flow Q-Sepharose anion exchange resin (Pharmacia Biotech AB) packed in an XK column (1.6 x 11 cm). The column was equilibrated with 2 column volumes (CV) of 1 M NaOH at a flow rate of 9 ml/min, followed by a water wash. Conductivity, pH and absorbency at 280 nm were monitored. When the conductivity dropped below 5 mS, the dried cleavage materials, dissolved in 7-8 M urea, were loaded onto the column and washed with 4 M urea. The unbound pure cationic peptide flowed through the column and was monitored as the leading peak. When the absorbance dropped to baseline, the bound material (*i.e.*, impurities) was washed off the column with 1 M NaOH and appeared as the second peak (Figure 9).

The flow-through peak was collected and pooled and the pH was adjusted to 7.0-7.5 with 0.2 N HCl. The sample was analyzed for purity by reverse phase HPLC (Figure 11), using a C8 column (4.6x10, Nova-Pak, Waters) and by acid-urea gel electrophoresis (West and Bonner, *Biochemistry 19*:3238, 1980). The identity of the MBI-11B7 peptide was confirmed by mass spectrometry to show that the flow through peak represents the homoserine form of the MBI-11B7 peptide.

EXAMPLE 13

UREA SEPARATION AND FURTHER PURIFICATION

The separation of the urea from the purified peptide utilized a high-throughput reverse phase chromatography technique by using the BioCAD™ (PerSeptive Biosystems Inc.) perfusion chromatography workstation and Poros® R-II 20 column, 4.6 x 100 mm (PerSeptive Biosystems Inc.). About 10 mg of the peptide were applied on the column at 5 ml/min, followed by equilibration of the column with

0.1% TFA. The peptide was eluted from the column by a gradient of increasing acetonitrile from 0 to 50% for 10 minutes at a flow rate of 5 ml/min. The peak of the further purified and urea free peptide was collected and lyophilized.

EXAMPLE 14

BACTERICIDAL ACTIVITY OF MBI-11B7CN PEPTIDE AND ITS HOMOSERINE/ HOMOSERINE LACTONE ISOFORMS

A comparison of anti-microbial activity between chemically and recombinantly synthesized cationic peptide was carried out.

The antimicrobial activities of the chemically synthesized MBI-11B7CN peptide and recombinant DNA synthesized MBI-11B7HSL (homoserine lactone form) and MBI-11B7HS (homoserine form) peptides were tested against various gram-negative and positive strains of bacteria, including antibiotic resistant strains. The *Agarose Dilution Assay* was performed as described in "Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically-Fourth Edition; Approved Standard" NCCLS document M7-A4 (ISBN 1-56238-309-4) Vol. 17, No 2 (1977).

The agarose dilution assay measures antimicrobial activity of peptides and peptide analogues, which is expressed as the minimum inhibitory concentration (MIC) of the peptides.

In order to mimic *in vivo* conditions, calcium and magnesium supplemented Mueller Hinton broth is used in combination with a low EEO agarose as the bacterial growth medium. Agarose, rather than agar, is used as the charged groups in agar prevent peptide diffusion through the media. The medium is autoclaved and then cooled to 50°C - 55°C in a water bath before aseptic addition of anti-microbial solutions. The same volume of different concentrations of peptide solution are added to the cooled molten agarose which is then poured to a depth of 3 - 4 mm.

The bacterial inoculum is adjusted to a 0.5 McFarland turbidity standard (PML Microbiological) and then diluted 1:10 before application on to the agarose plate. The final inoculum applied to the agarose is approximately 10^4 CFU in a 5 - 8 mm diameter spot. The agarose plates are incubated at 35°C - 37°C for 16 to 20 hours.

The MIC is recorded as the lowest concentration of peptide that completely inhibits growth of the organism as determined by visual inspection. Representative MICs for the cationic peptides against various bacterial strains are shown in Table 3.

TABLE 3

MINIMUM INHIBITORY CONCENTRATION (MIC) VALUES FOR MBI-11B7CN (CARBOXY-AMIDATED), MBI-11B7HSL (HOMOSERINE LACTONE FORM) AND MBI-11B7HS (HOMOSERINE FORM) PEPTIDES, AGAINST VARIOUS GRAM-NEGATIVE AND GRAM-POSITIVE BACTERIA STRAINS

Organism	Organism #	Source	MIC (g/ml)		
			11B7CN 92A1	11B7HSL 203B1	11B7HS 203B1
A. calcoaceticus	AC2	ATCC	2	4	2
E. cloacae	ECL7	ATCC	>64	>64	>64
E. coli	ECO5	ATCC	8	8	32
K. pneumoniae	KP1	ATCC	8	8	32
P. aeruginosa	PA4	ATCC	>64	>64	>64
S. maltophilia	SMA2	ATCC	32	32	64
S. marcescens	SMS3	ATCC	>64	>64	>64
E. faecalis	EFS1	ATCC	2	1	2
E. faecalis	EFS8	ATCC	16	16	32
S. aureus	SA14	Bayer	4	1	2
S. aureus	SA93	Bayer	1	1	1
S. epidermidis	SE10	Chow	2	4	8

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that various modifications may be made to the disclosed

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